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Prolonged REM sleep restriction induces metabolic syndrome-related changes: Mediation by pro-inflammatory cytokines

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ABSTRACT

Chronic sleep restriction in human beings results in metabolic abnormalities, including changes in the control of glucose homeostasis, increased body mass and risk of cardiovascular disease. In rats, 96 h of REM sleep deprivation increases caloric intake, but retards body weight gain. Moreover, this procedure increases the expression of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which may be involved with the molecular mechanism proposed to mediate insulin resistance. The goal of the present study was to assess the effects of a chronic protocol of sleep restriction on parameters of energy balance (food intake and body weight), leptin plasma levels and its hypothalamic receptors and mediators of the immune system in the retroperitoneal adipose tissue (RPAT). Thirty-four Wistar rats were distributed in control (CTL) and sleep restriction groups; the latter was kept onto individual narrow platforms immersed in water for 18 h/day (from 16:00 h to 10:00 h), for 21 days (SR21). Food intake was assessed daily, after each sleep restriction period and body weight was measured daily, after the animals were taken from the sleep deprivation chambers. At the end of the 21 day of sleep restriction, rats were decapitated and RPAT was obtained for morphological and immune functional assays and expression of insulin receptor substrate 1 (IRS-1) was assessed in skeletal muscle. Another subset of animals was used to evaluate blood glucose clearance. The results replicated previous findings on energy balance, e.g., increased food intake and reduced body weight gain. There was a significant reduction of RPAT mass ($p < 0.001$), of leptin plasma levels and hypothalamic leptin receptors. Conversely, increased levels of TNF-α and IL-6 and expression of phosphorylated NFκ-β in the RPAT of SR21 compared to CTL rats ($p < 0.01$, for all parameters). SR21 rats also displayed reduced glucose clearance and IRS-1 expression than CTL rats ($p < 0.01$). The present results indicated that 21 days of sleep restriction by the platform method induced metabolic syndrome-related alterations that may be mediated by inflammation of the RPAT.

1. Introduction

The past decades have witnessed a major change in sleep habits in numerous countries, especially industrialized ones, with a chronic and meaningful reduction of sleep hours (Di Milia et al., 2013; Kim et al., 2013; Nagai et al., 2013; Xiao et al., 2013). Findings from epidemiologic studies indicate that disturbed- and short periods of sleep are a risk factor for obesity (Moraes et al., 2013), diabetes (Togeiro et al., 2013), cardiovascular disease (Rod et al., 2014) and metabolic syndrome (Drager et al., 2013). Controlled laboratory studies have also shown several behavioral and physiological alterations that seem to favor the induction of metabolic syndrome (Chaput et al., 2008; Van Cauter et al., 2008), including reduction of leptin and increase of ghrelin levels (Spiegel et al., 1999, 2004), which may explain the augmented caloric intake (Beebe et al., 2013; Hogenkamp et al., 2013) and craving for carbohydrate- and fat-rich foods observed in volunteers (Nedeltcheva et al., 2009; Spiegel et al., 2004), possibly reflecting a homeostatic and hedonic response to this situation (Taheri et al., 2004). In addition, increased cortisol levels and sympathetic tonus (Spiegel et al., 1999), reduced glucose clearance and increased insulin resistance (Donga et al., 2010) have been found in short-term sleep restriction laboratory studies. These changes seem to be interconnected, since adrenalectomy reduces body weight in leptin deficient mice (Dubuc and Wilden, 1986) and dexamethasone, a glucocorticoid receptor agonist, increases serum levels of leptin (Dagogo-Jack et al., 1997; Laferrère et al., 2002, 2000), suggesting that these

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hormones participate in the regulation of leptin function (Bose et al., 2009).

Adipocytes of sleep-restricted subjects show reduced insulin signaling, with less phosphorylation of the kinase b protein (pAkt) and less glucose uptake by the adipose tissue (Broussard et al., 2012). Despite the evidence presented by controlled studies, clinical populations provide contradictory results, with primary insomniac patients presenting lower metabolic rate (Bonnet and Arand, 2003), and obstructive sleep apnea patients, higher resting metabolic rate than healthy volunteers (Ucok et al., 2011). In addition, a thorough review paper concluded that numerous limitations of studies in human beings preclude drawing of causality between short sleep and changes in energy metabolism (Klingenberg et al., 2012). Therefore, animal models of sleep restriction could be a more useful approach to investigate possible causal relationships and the mechanisms involved in metabolic syndrome-related changes.

In rodents, sleep deprivation has produced results somehow similar to human studies regarding the endocrine changes, including reduction of anabolic (Andersen et al., 2005; Everson and Crowley, 2004; Everson and Nowak, 2002; Hipolide et al., 2006; Venâncio et al., 2012) and increase of catabolic hormones (Galvão et al., 2009; Rosa Neto et al., 2010; Suchecki et al., 1998). This endocrine unbalance seems to be a determining factor for the sleep deprivation-induced body weight loss (Koban and Stewart, 2006; Suchecki et al., 2003; Suchecki and Tufik, 2000) despite the hyperphagia displayed by the animals (Galvão et al., 2009; Hipolide et al., 2006; Koban et al., 2008; Koban and Swinson, 2005; Martins et al., 2010). Moreover, REM sleep-deprived rats exhibit increased energy expenditure, measured either directly (Koban and Swinson, 2005) and indirectly (Hipolide et al., 2006). Increased food intake appears to be a consequence of increased NPY gene expression (Martins et al., 2010) and protein synthesis (Koban et al., 2008), in addition to increased orexin expression (Galvão et al., 2009), most likely due to low leptin levels (Everson and Crowley, 2004; Koban and Swinson, 2005; Moraes et al., 2014), resulting from reduced fat tissue (Hipolide et al., 2006). Moreover, increased expression of IL-6 and TNF-α has been reported in the white adipose tissue of rats submitted to REM sleep deprivation (Rosa Neto et al., 2010), indicating that, even though this protocol induces loss of fat tissue, disruption of normal sleep leads to increased inflammation.

Sleep restriction, rather than deprivation, reflects better the human condition and is a useful tool to disclose the mechanisms involved in the effect of reduced sleep hours on metabolic profile. Findings from sleep restriction studies in rodents reproduce many, but not all, metabolic changes induced by sleep curtailment in humans. For instance, rats submitted to eight days of sleep restriction or to sleep disturbance in rotating drums exhibit impairment of glucose clearance and reduced insulin levels, after a bolus injection of glucose (Barf et al., 2010). In an interesting experimental protocol, Barf and co-workers exposed rats to the rotating drums for five days per week interspersed by two days in the home-cage (mimicking a human week, with sleep restriction during "working" days and free sleep time during the "weekends") and found that during the "weekend" rats ate less, but gained more weight, compared to the "working" days. Insulin and leptin levels were reduced during the latter and normalized during the former period (Barf et al., 2012). However, 10 days of 18 h of forced wakefulness in rotating wells does not change oxygen consumption compared to control rats, indicating that sleep restriction does not alter the rate of energy expenditure (Caron and Stephenson, 2010). Sleep restriction for 21 days, induced by the platform method (18 h of sleep deprivation/6 h of sleep allowance), does not produce the same changes as sleep deprivation, insofar as sleep-restricted rats do not exhibit differences from controls in body weight gain, food intake, lipid profile and glucose metabolism (Martins et al., 2010). However, in this study, animals were fed a balanced liquid diet, to which they were adapted to, preventing food waste and likely optimizing food utilization. More recently, a non-instrumental approach to induce sleep restriction in rats, namely ablation of the ventrolateral preoptic area, resulted in slower weight gain and no changes in metabolic parameters, raising the question of whether or not shorter sleep hours are indeed related to metabolic syndrome (Vetrivelan et al., 2012).

One of the mechanisms proposed to link obesity to metabolic syndrome in humans involves inflammation of the adipose tissue (for review see (Aroor et al., 2013; Sun et al., 2012; Yao et al., 2014)). Sleep apnea patients exhibit elevation of inflammatory markers, such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) (Teramoto et al., 2004, 2005) and these abnormalities can be reversed by continuous positive airway pressure (Coughlin et al., 2007; Teramoto et al., 2008) and weight loss (Sahlman et al., 2012). Interestingly, sleep fragmentation, which is one of the major characteristic of sleep apnea, induces insulin resistance in epididymal fat pads of mice, by means of induction of the enzyme NADPH oxidase 2 and infiltration of macrophages in visceral adipose tissue. This effects were absent or greatly attenuated in knockout mice for this enzyme (Zhang et al., 2014).

To the best of our knowledge, there are no study yet published on the effect of prolonged sleep restriction on the immunological profile of rats, and whether or not this could constitute a mechanism for the development of metabolic changes reminiscent of metabolic syndrome in humans. Based on the abovementioned data, we hypothesized that prolonged REM sleep restriction would induce metabolic syndrome-related changes in parallel to increased inflammatory markers measured in white adipose tissue.

2. Methods

2.1. Animals

Thirty-four 90-day old Wistar male rats were used. They were provided by Centro de Desenvolvimento de Modelos Experimentais (CEDEME) of Universidade Federal de São Paulo and maintained in the experimental room, under controlled temperature (21 ± 1 °C) and 12 h light–dark cycle with lights on at 7:00 h. Throughout the study, food and water were provided ad libitum. All experimental procedures were carried out in accordance to the institutional and Brazilian guidelines for the ethical use of laboratory animals and were approved by UNIFESP Ethical Committee (CEP# 0334/11).

2.2. REM sleep restriction protocol

Sixteen rats were submitted to REM sleep restriction for 21 days (SR21), by placing them onto individual 6.5 cm platforms, immersed in water until 1 cm of the upper surface (Fig. 1). Everyday these animals were placed in the water containers at 16:00 h and remained there until 10:00 h, when they were removed and placed back in their home-cages, resulting in 18 h/day of sleep disruption. This method suppresses REM sleep and induces approximately 25% reduction of slow wave sleep, during the 18 h period in the water container, in addition to numerous awakening episodes (Machado et al., 2005), recapitulating some aspects of the sleep pattern of obstructive sleep apnea patients (Bittencourt et al., 2001). The 6 h period that rats are allowed to sleep in their home-cages is not sufficient to compensate for the sleep loss, since major REM sleep rebound is observed in the first 24 h after the end of the protocol (Machado et al., 2005).

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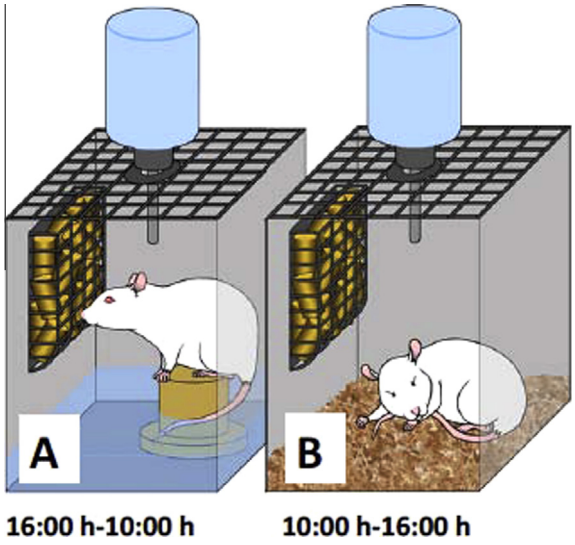

Fig. 1. Experimental model of the single platform sleep deprivation. Rats of sleep restriction group were maintained for 18 h/day in a cage containing a single platform immersed in water (A) and then transferred to a cage containing wood shave and maintained for 6 h/day (B) Control rats were kept in the cage containing dry bedding throughout the entire protocol.

Eighteen rats in the control group (CTL) remained in the same room as SR21 rats, housed individually in the same water container, filled with cage bedding, instead of water, where they slept freely. Three days before the onset of the experimental protocol, rats were adapted to their environments for 1 h/day, always at 16:00 h.

2.3. Assessment of body weight and food intake

Body weight was measured daily, from the adaptation period until the last day of sleep restriction and in the corresponding days for CTL rats. On the first day of the protocol, 150 g of chow was offered and, from then on, the chow was weighed every day, at 10:00 h (food intake during sleep restriction period) and 16:00 h (food intake during sleep allowance period). The amount of food ingested was calculated by 100 g of body weight and summed up per week.

2.4. Spillage collection

At the same time that the chow remained in the food containers was assessed, all the debris in the bottom of the water container was collected, separated from feces and dried in sterilizer for 24 h, at 50 °C and the amount obtained was subtracted from the food removed from the food containers. Therefore, the chow ingested was estimated, as closed as possible, to reality.

2.5. Plasma leptin levels

At 10:00 h of the 21st day of the protocol (or the corresponding day for CTL group), rats were decapitated and trunk blood was collected in EDTA-containing vials (BD Vacutainer®) and centrifuged at 2300 rpm, at 4 °C for 20 min. Plasma was extracted and maintained at −20 °C until determination of leptin levels by a commercial ELISA kit (ABCAM, Cambridge, UK), specific for rats, with sensitivity lower than 30 pg/mL. The intra- and inter-assay variability is 10% and 12%, respectively. The assay was carried out according to the vendor's protocol.

2.6. Tissue extraction and measurement of adipose tissue mass

Retroperitoneal adipose tissue (RPAT) was chosen because it is very poor in brown adipose tissue, which could have influenced the metabolic response, given its high mitochondrial density. The tissue was obtained at 10:00 h of Day 21 of sleep restriction or at the same time for CTL rats. The tissue was weighed and divided in two fragments. One was frozen in liquid nitrogen and stored in −80 °C until evaluation, whereas the other was immersed in 4% paraformaldehyde, included in paraphin and sliced in 3 μm thick slices in a microtome and stained in hematoxilin and eosin solution (HE staining). Digital images of the HE-stained sections were captured using an Olympus brightfield microscope BX50, camera DP71 (Melville, NY) with a 40 × objective. A blind analysis of the cross-sectional areas of 100 fibers per adipose tissue sample was performed by DPV using the software Axio Vision 4.6 (Carl Zeiss MicroImaging GmbH).

2.7. Protein extraction of RPAT and skeletal muscle

RPAT and the gastrocnemius muscle were homogenized using Ultra Turrax T 25 basic homogenizer (Ika, Wilmington, NC, USA), at 6000 rpm for 2 min with buffer extraction, containing protease and phosphatase inhibitors (Pierce Scientific, Rockford, Ilinios, USA). The homogenate was maintained at 4 °C for 30 min and centrifuged at 15,000 × g, 4 °C for 10 min. This step was repeated once more and the final supernatant was collected and the protein concentration was measured by the Lowry method (Lowry et al., 1951), using the Bio-Rad Protein Assay kit (Bio-Rad, Richmond, VA, EUA). A bovine albumin solution (1 mg/mL) was used as standard. The absorbance was assessed in a microtitter reader (Biotrak II, Amersham Biosciences), using 750 nm filters. The supernatant was aliquotted and stored at −80 °C until being used. Samples

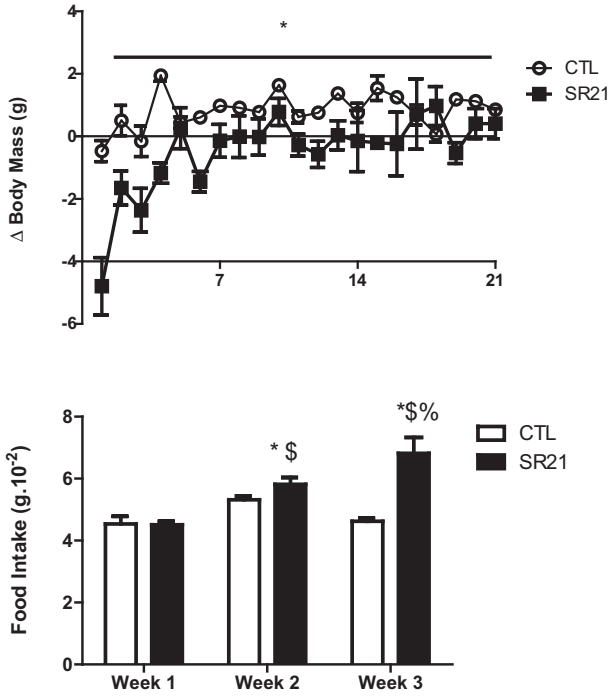

Fig. 2. Effect of sleep deprivation on body mass (g) and food intake (g of food/100 g of body weight). Body mass (A) was evaluated daily and food intake (B) was assessed daily, but is represented week by week in control (CTL) and in sleep restricted rats (SR21). $^{*}p < 0.05$ compared to CTL group; $^{\$}p < 0.05$ compared to respective week 1;% $p < 0.05$ compared to respective week 2. Data are presented as mean ± S.E.M of 12 rats/group.

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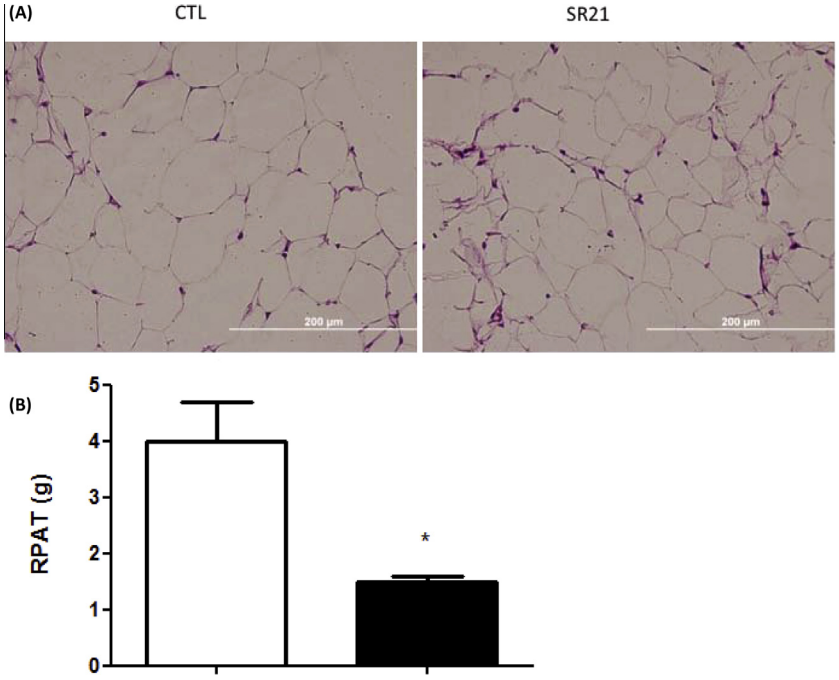

Fig. 3. Effects of sleep restriction on the inflammatory infiltrate and on the mass of the retroperitoneal adipose tissue (RPAT). Macrophage infiltrate in the adipose tissue (A) and RPAT were analyzed in both control (CTL; white bar) and SR21 rats (black bar). Data are expressed as mean ± S.E.M. of 6 rats/group. $^{*}p < 0.001$ compared to CTL group.

obtained by this method were used for both western blotting and ELISA analysis.

2.8. Western blotting for Toll Like Receptor 4 (TLR4) and phosphorylated NFkB (pNFkB) in RPAT, phosphorylated Insulin Receptor Substrate-1 (pIRS-1) in skeletal muscle and leptin receptor (Obr) in the hypothalamus

Total cell lysates of the RAPT and gastrocnemius skeletal muscle were homogenized in lysis buffer (T-PER Tissue Protein Extraction Reagent, Thermo Scientific, USA and protease and phosphatase inhibitors cocktails, Pierce, USA). The total protein concentration was determined by Lowry method (Gardner et al., 2005). The total cell lysates was analyzed in SDS–polyacrylamide gel electrophoresis. The separating gel was prepared in 0.4 M Tris–HCl buffer (pH 8.8), containing 10% polyacrylamide, 0.1% SDS, 0.01% TEMED and 0.05% ammonium persulfate. The stacking gel was prepared in 0.1 M Tris–HCl buffer (pH 6.8) containing 3% polyacrylamide, 0.1% SDS, 0.01% TEMED and 0.05% ammonium persulfate. An aliquot of 75 µg of RAPT extract was mixed with denaturant and reductant buffer, composedof 0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% bromophenol blue and 4% mercaptoethanol. Samples were heated at 95 °C for 5 min and applied on the gel. The running buffer was made of Tris–HCl (0.025 M) containing 0.18 M glycine, pH 8.3, and 1% SDS. The run was performed at 100 V for 2.5 h at room temperature. After the electrophoresis, samples were transferred to a nitrocellulose membrane. The blockade was made for 2 h at room temperature, under slow agitation, with a buffer containing 10 mM Tris–HCl, 150 mM NaCl, pH 7.5 (TBS) and 5% nonfat milk. The membrane was washed twice with TBS containing 5% of Tween 20, and incubated with anti-TLR4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NFκ-β p50 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), for RAPT samples, and anti-IRS-1p (Abcam, Cambridge, UK) for skeletal muscle samples. The primary antibodies were diluted at 1:1000. The membrane wash was made with TBS containing 0.05% Tween 20, for 10 min. This step was repeated three times and was followed by incubation with Alexa Fluor goat anti-rabbit antibody 680 nm wavelength (1:10,000 dilution; Invitrogen), incubated for 60 min at room temperature prior to washing with TBS-T. Visualization and quantification was carried out with the LI-COR Odyssey® scanner and software (LI-COR Biosciences Inc. Lincoln, NE, USA).

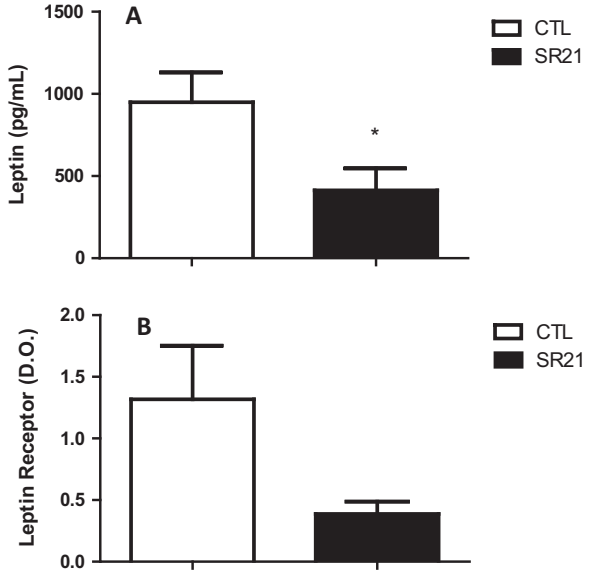

Fig. 4. Effects of sleep restriction on plasma leptin levels (pg/mL) and protein expression of leptin receptor in hypothalamic extracts. Leptin levels were measured in the plasma of CTL and SR21 groups (A). The protein expression of leptin receptor was accessed by western blotting, at the end of sleep restriction period (B). Data are presented as mean ± S.E.M. $^{*}p < 0.001$ compared to CTL group ($n = 6$ rats/group in A and $n = 4$ rats/group in B).

2.9. Quantification of IL-6, IL10 and TNF-α in RPAT

The sample of RPAT was used in western blot and ELISA assays. Quantitative assessment of TNF-a, IL-6 and IL-10 was carried out

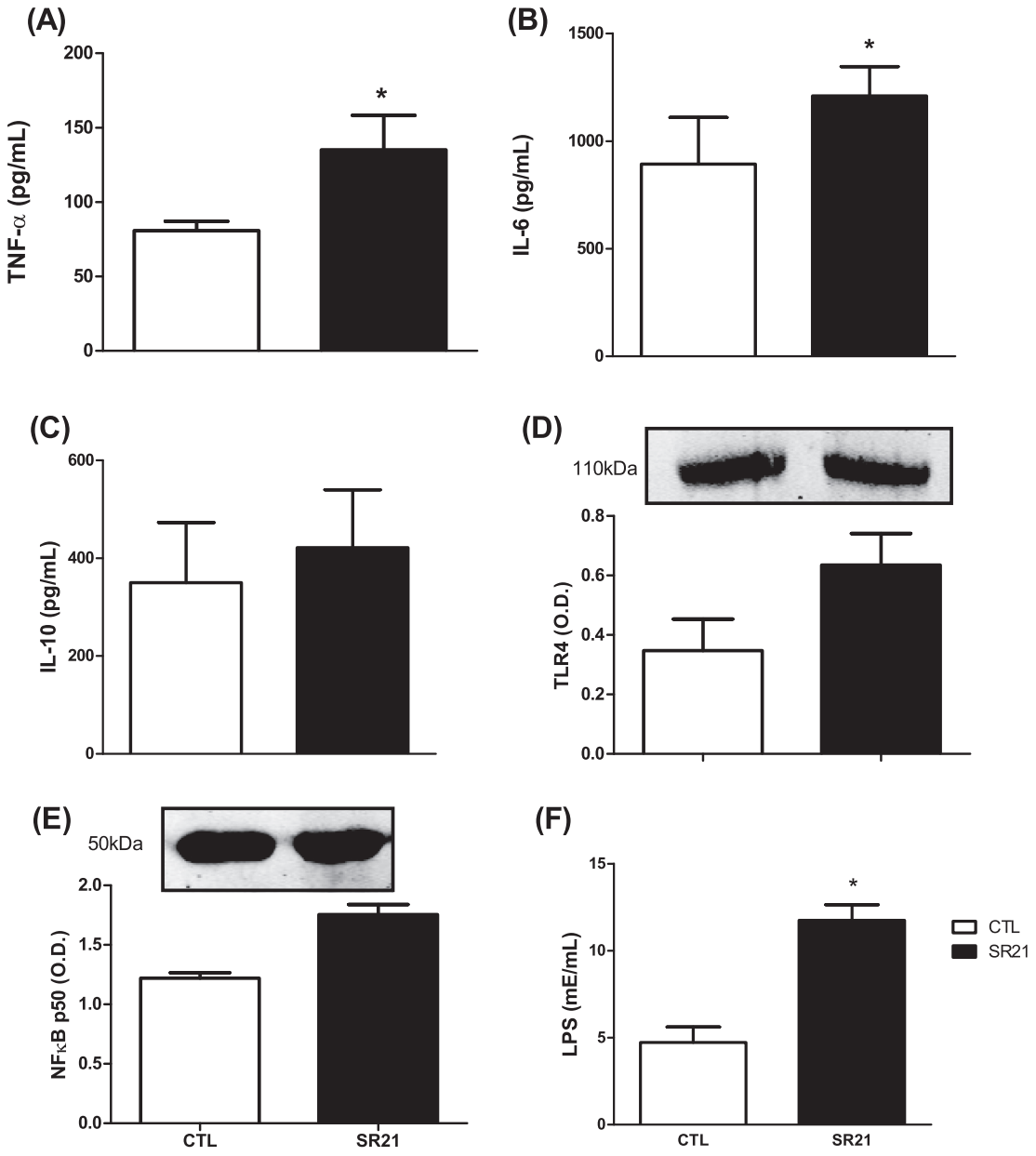

Fig. 5. Effects of sleep restriction on cytokine production in the RPAT and plasma endotoxin levels. TNF-α (pg/mL) (A), IL-6 (pg/mL) (B) and IL-10 (pg/mL) (C), Toll-like receptor 4 (TLR4) expressed in optic density (O.D) (D), phosphorylated NF-κB (pNF-κB, O.D.) (E) and endotoxin (mE/mL) (F). Cytokines were measured by ELISA; TLR4 and pNF-κB expression was assessed by western blotting, and endotoxin levels were measured in the plasma of CTL and SR21 groups. Data are represented by mean ± S.E.M. of 6 rats/group. $^{*}p < 0.001$ compared to CTL group.

by ELISA (DuoSet ELISA, R&D Systems, Minneapolis, MN). For the TNF-α, IL-6 and IL-10 assays, the sensitivity was 5.0 pg/mL. Intra- and inter-assay variability of the TNF-α and IL-6 kits was 2.7–5.2% and 4.9–9.5%, respectively. Assay sensitivity for IL-10 was 10 pg/mL. The intra-assay and inter-assay variability of the IL-10 kit was 2.0–4.2% and 3.3–6.4%, respectively. All samples were run in duplicates, and the mean values were reported.

2.10. Circulating endotoxin levels

The method used here was described by Lira and colleagues (Lira et al., 2012), using a chromogenic Limulus Amebocyte Lysate (LAL) test, which is a quantitative test for Gram-negative bacterial endotoxin (Cambrex Corporation, 8830 Biggs Ford Road, Walkersville–USA). Briefly, this test is based on the activity of a proenzyme present in LAL that is directly proportional to the endotoxin levels present in a sample. Once active, this enzyme cleaves a substrate, leading to a p-nitroaniline (pNA) liberation. The photometrical measurement was made at 405–410 nm at the end of the reaction. The correlation between the absorbance and the endotoxin concentration is linear in the range of 0.1–1.0 EU/mL. All samples were run in duplicate within the same plate.

2.11. Intraperitoneal glucose tolerance test

After sleep restriction, a bolus of glucose (2 mg of glucose per g of body weight) was administered to another subset of rats (6 CTL and 4 SR21 rats). Blood samples were collected from the lateral tail vein before (basal) and 10, 30, 60 and 70 min after glucose administration. Glucose levels were measured using a manual glucosimeter Accu-Chec® (Roche Diagnostics). The area under the curve

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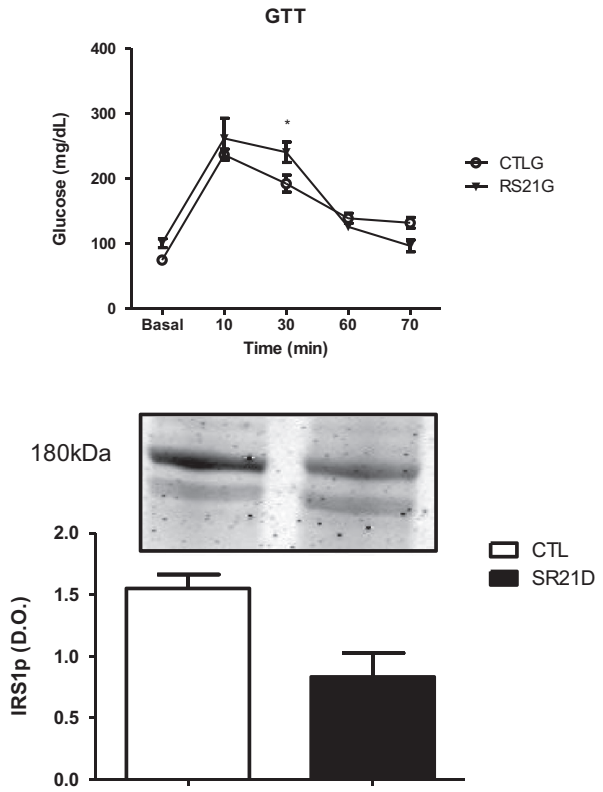

Fig. 6. Effects of sleep restriction on the glucose clearance and on phosphorylated IRS-1 protein expression in the extract of skeletal muscle tissue. Glucose Tolerance Test was performed to examine the glucose clearance in CTL and SR21 groups at 0, 10, 30, 60 and 70 min after an i.p. glucose bolus administration (A). The protein expression of the pIRS-1 protein in muscle extract was accessed by western blotting at the end of the sleep restriction period (B). Data are presented as mean ± S.E.M. $^{*}p < 0.001$ compared to CTL group ($n = 6$ rats/group in A and $n = 4$ rats/group in B).

(AUC) for blood glucose levels was calculated by the trapezoid formula (Pruessner et al., 2003).

2.12. Statistical analysis

Data on food intake were analyzed by two-way ANOVA for repeated measures, with group (CTL, SR21) and week (week 1, week 2, week 3), whereas variation of body weight was analyzed by ANOVA for repeated measures, with group and day (basal, day 1–day 21) as main factors. Moreover, average weight variation during each week was analyzed by two-way ANOVA for repeated measures with group and week as main factors. Blood glucose levels were analyzed by two-way ANOVA for repeated measures with group and time-point (basal, 10, 30, 60, 70 min) as main factors. *Post-hoc* analyses were done by the Newman-Keuls test. Pairwise comparisons were carried out by the Student's *t* test. Correlations between RPAT mass and immunological parameters were carried out by Pearson's correlation test. Significant results were considered when $p < 0.05$.

3. Results

3.1. Body weight variation and food intake

There was no difference in body weight between the groups at the onset of the protocol (CTL = 331.83 ± 25.34; SR21 = 343.82 ± 22.44; $t_{21} = 1.19$; $p = 0.25$). There was an interaction between Day and Group [$F_{(20,400)} = 4.374$; $p < 0.001$]. Analysis of this interaction showed that, compared to CTL group, RS induced a robust weight loss on Day 1 ($p < 0.00004$), Day 3 ($p < 0.05$) and Day 4 ($p < 0.003$). While CTL rats maintained a stable body weight, SR21 rats lost more weight on Days 1 ($p < 0.005$) and 3 ($p < 0.05$). After that, there was no significant weight variation in this group (Fig. 2A). Analysis of the weekly variation of body weight revealed that SR21 rats lost more weight in the first week, but subsequently there was a recovery to baseline values (p's < 0.001 compared to week 2 and week 3, which did not differ from each other), whereas for CTL rats, there was a continuous increase from week 1 to week 3 (p's < 0.001). Therefore, CTL rats gained more weight than SR21 ones (p's < 0.01), as indicated by an interaction between group and week [$F_{(2,44)} = 32.435$; $p < 0.0001$].

There was an interaction between Week and Group [$F_{(2,16)} = 19.289$; $p < 0.0001$] for food intake. SR21 rats consumed more chow than CTL rats on week 3 ($p < 0.0003$). CTL rats ate more on week 2 than on the other periods (p's < 0.03), whereas SR21 rats exhibited a progressive increase of chow intake (week 1 < week 2 < week 3; p's < 0.002). These results are presented in Fig. 2B.

3.2. RPAT mass

Fig. 3A shows a representative micrograph of RPAT of CTL and SR21 rats. Sleep restriction produced a significant reduction of RPAT (t10 = 3.533; $p < 0.02$; Fig. 3B).

3.3. Plasma leptin levels and expression of hypothalamic Obr

There was a robust reduction of leptin levels after 21 days of sleep restriction (t10 = 2.374; $p < 0.04$). Although not statistically significant, expression of hypothalamic Obr showed a trend to reduction in SR21, compared to CTL rats (t6 = 2.084; $p = 0.08$). These results are shown in Figs. 4A and 4B.

3.4. Plasma levels of pro-inflammatory cytokines, Western blotting for TLR4 and pNFB, and endotoxin plasma levels

Prolonged sleep restriction increased plasma levels of TNF- (t10 = 6.405; $p < 0.0001$) and IL-6 (t10 = 7.514; $p < 0.0001$), but not IL-10, compared to CTL group (Figs. 5A, 5B and 5C, respectively). There was no change in the expression of TLR4 ($p < 0.1$; Fig. 5D), but pNFB was augmented in the RPAT of SR21 rats compared to that of CTL ones (t6 = 5.596; $p < 0.002$; Fig. 5E). In addition, increased levels of plasma endotoxin were also detected in SR21 rats, compared to CTL ones (t10 = 6.718; $p < 0.0001$; Fig. 5F).

3.5. Intraperitoneal glucose tolerance test and pIRS-1

ANOVA detected an interaction between group and time-point [$F_{(4,40)} = 4.199$; $p < 0.007$]. Analysis of this interaction revealed that both CTL and RS rats exhibited peak blood glucose 10 min after the glucose administration (p's < 0.01). However, in CTL rats, glucose levels began to return to basal at the 30 min time-point (Basal < 10 min > 30 min > 60 min = 70 min; p's < 0.01). For SR21 rats, glucose levels remained elevated at the 30 min time-point and return to basal levels occurred only at 60 min (Basal < 10 - min = 30 min > 60 min = 70 min; p's < 0.002). Glucose levels at 30 min were higher in SR21 than in CTL rats ($p < 0.04$). Comparison of basal glucose levels (pre-glucose bolus injection) revealed that RS21 rats displayed higher values than CTL rats (t10 = 3.519; $p < 0.006$), whereas sleep restricted rats also exhibited higher glucose levels throughout the test (AUC of CTL = 5738.75 ± 294.92 mg/dL/70 min × AUC of SR21 = 13,460.83 ± 2230.30 mg/dL/70 min; t8 = 6.749; $p < 0.0002$). Compared to CTL rats, SR21 showed reduction of IRS-1 phosphorylation in the skeletal muscle (t6 = 3.212; $p < 0.001$). These results can be seen in Figs. 6A and 6B.

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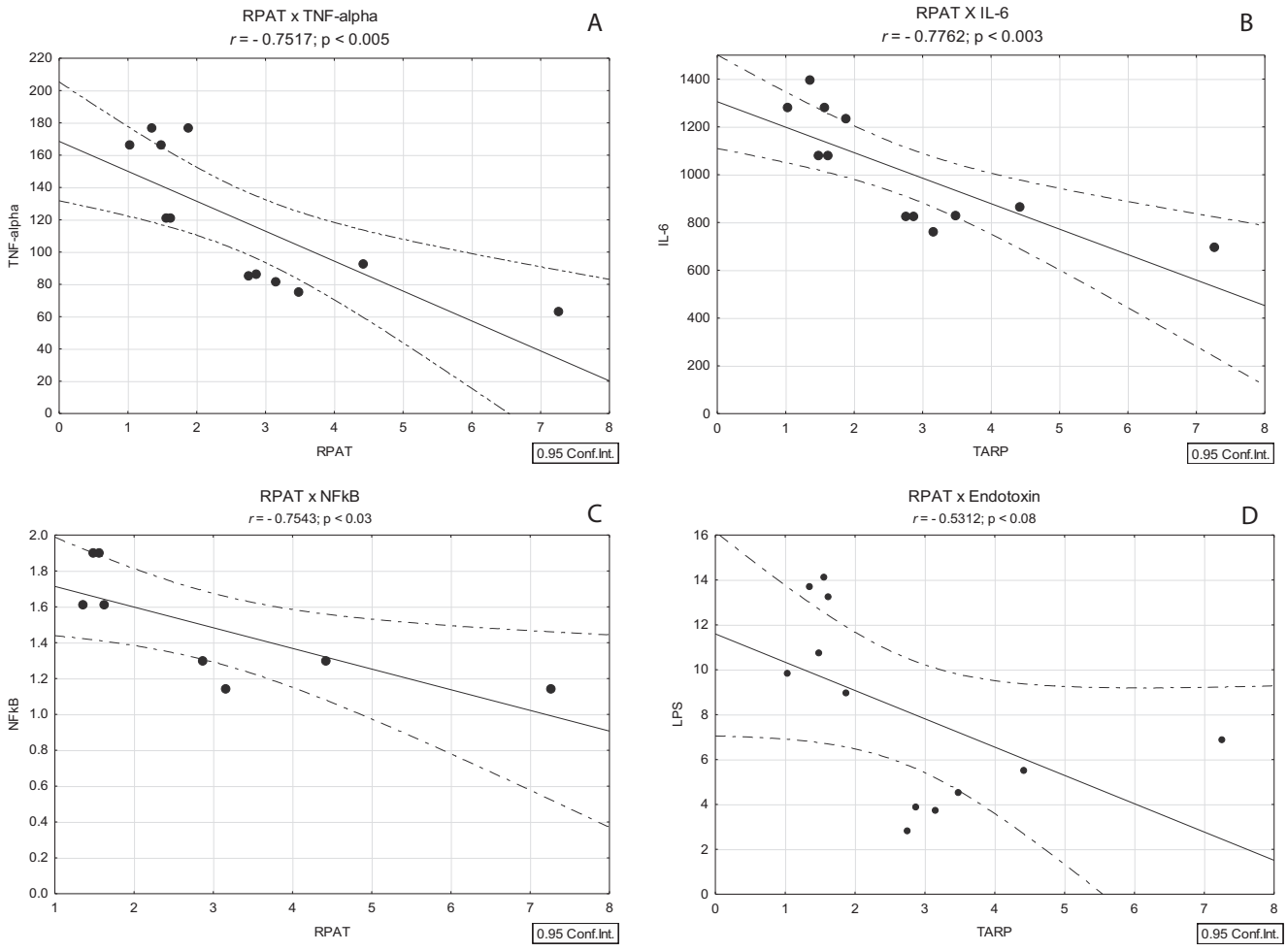

Fig. 7. Scatterplots of the correlations between retroperitoneal adipose tissue mass and TNF-α (A), IL-6 (B), pNF-κB (C) and endotoxin levels (D). Correlations were performed by the Pearson's correlation test.

3.6. Correlations between RPAT and immunological parameters

Negative correlations were found between RPAT and TNF-α ($r = -0.7517$; $p < 0.005$; $N = 12$), RPAT and IL-6 ($r = -0.7762$; $p < 0.003$; $N = 12$) and RPAT and NFB ($r = -0.7543$; $p < 0.03$; $N = 12$), the correlation between RPAT and endotoxin levels were close to significant ($r = -0.5312$; $p < 0.08$; $N = 12$). These results are displayed in Fig. 7.

4. Discussion

The present study showed that prolonged sleep restriction resulted in an abrupt loss of body weight in the first day of the protocol and reduction of retroperitoneal adipose tissue. This effect of sleep restriction occurred despite the greater food intake of rats while in the restriction chamber. This pattern of greater energy expenditure, represented by increased food intake and loss of body weight has been well documented in sleep deprivation paradigms, irrespective of the kind of protocol used (Bergmann et al., 1989; Elomaa, 1979; Everson and Crowley, 2004; Koban and Swinson, 2005; Suchecki et al., 2003). The results of body weight variation and food intake indicate that progressive increase of chow consumption compensated for the intense weight loss observed in the very beginning of the sleep restriction protocol. Previously, a similar result, e.g., no difference in body weight on the last day of sleep restriction, was obtained with a liquid diet (Martins et al., 2010).

Reduction of RPAT (Rosa Neto et al., 2010) and of total fat body composition (Hipolide et al., 2006) is also observed in 96 h REM sleep deprived rats, indicating that fat metabolism may contribute to energy production in this paradigm. Mobilization of fat tissue to this end seems to take place with greater intensity in the beginning of the sleep restriction period, which coincides with increased catecholamine-induced lipolysis and uncoupling protein 1 (UCP1) expression (Koban and Swinson, 2005). After the first four days of sleep restriction the lipolytic effect seemed to have faded away, since the weight loss was halted and rats began to gain weight, albeit at a slower rate than CTL rats. Nonetheless, SR21 rats still exhibited less RPAT than CTL rats on the last day of sleep restriction, which most likely explains lower leptin plasma levels, a hormone released by adipocytes that signals energy stores and regulates the activity of neuropeptides that control hunger and satiety (Klok et al., 2007). Similar results on leptin levels were described in sleep-deprived rats (Everson and Crowley, 2004; Koban and Swinson, 2005; Moraes et al., 2014) and sleep-restricted human beings (Spiegel et al., 2004). Interestingly, the present study showed that leptin receptors in the hypothalamus were not upregulated as a consequence of reduced levels of the hormone. In fact, these receptors were also reduced, albeit at a non-significant level,

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indicating that under this situation, there is no compensatory regulation in the leptin signaling, which could explain the progressive increase in food intake exhibited by sleep-restricted rats.

Even though RPAT was smaller in SR21 rats, the expression of pro-inflammatory cytokines as well as pNFκB in this tissue was higher in this group than in control rats. Several studies show a positive association between sleep deprivation or sleep restriction and circulating pro-inflammatory cytokines, both in humans and animals (Ashley et al., 2013; Axelsson et al., 2013; Chennaoui et al., 2011; Everson, 2005). In the RPAT, the present results corroborated a previous study showing that 96 h of REM sleep deprivation increased the expression of IL-6 and TNF-α (Rosa Neto et al., 2010). Moreover, the increase in pNFκB in the adipose tissue indicates that this is the site of cytokines production, since pNFκB dissociates from the beta subunit of the Inhibitor of κB kinase complex (IKKβ) and translocates to the nucleus, inducing the transcription of inflammatory genes (for review see (Baker et al., 2011)).

TNF-α production is related to activation of M1-type macrophages, which are positively associated to tissue inflammation and insulin resistance (Aouadi et al., 2013). Macrophage infiltration induces glucose tolerance and insulin resistance (Nishimura et al., 2009), whereas macrophage silencing by interference RNA reduces the production of inflammatory mediators and increases tissue sensitivity to insulin (Aouadi et al., 2013). In the skeletal muscle, TNF-α decreases IRS-1 phosphorylation and dextro-glucose uptake explaining the mechanism by which TNF-α induces insulin resistance and obesity (del Aguila et al., 1999). In healthy volunteers, 4 nights of 4.5 h of sleep results in less kinase B (Akt) phosphorylation, a protein closely involved in the mechanism of tissue glucose uptake (Broussard et al., 2012). Therefore, increased basal glucose levels and reduced glucose clearance after 21 days of SR might have resulted from lower expression of proteins involved in insulin receptor signaling in the skeletal muscle, such as ISR-1 (shown in the present study) and Akt in the adipose tissue (Broussard et al., 2012).

Increased endotoxin levels was seen after prolonged sleep restriction, in agreement with findings of prolonged sleep deprivation (Everson, 2005). Endotoxemia is caused by lipopolysaccharide (LPS), a component of gram-negative bacteria, which appears to originate from the intestine; SR may have induced compartment changes in these molecules, by increasing the permeability of intestinal tight junctions (Teixeira et al., 2012). LPS also acts as an agonist of TLR4, which are involved in the innate immune response, such as activation of macrophages and monocytes, activating NFkB and synthesis of pro-inflammatory cytokines. The expression of TLRs 2 and 4 in monocytes surface is increased in patients with obstructive sleep apnea (Akinnusi et al., 2013), which is involved with increased production of IL-6, TNF-α and interferon-γ (Yamauchi et al., 2006), whereas those treated by continuous positive airway pressure display reduced expression of these receptors and of pro-inflammatory cytokines (Akinnusi et al., 2013; Yamauchi et al., 2006).

In the present study, we were unable to recapitulate all characteristics of metabolic syndrome, such as obesity. One possibility is that rats consumed regular chow, whereas human beings tend to consume hypercaloric food (Nedeltcheva et al., 2009; Spiegel et al., 2004); alternatively, it is possible that longer periods of sleep restriction are necessary to produce the entire feature of metabolic syndrome. Despite these drawbacks, SR21 induced changes that resemble those seen in metabolic syndrome, such as increased glycemia, and insulin resistance. Interestingly, these outcomes are likely to have been mediated by inflammation of the RPAT. We believe that this paradigm can be useful to study the central mechanisms involved in sleep restriction-induced metabolic syndrome, given the unfeasibility to do it in human beings.

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